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Biodegradation Potentials of *Aspergillus sydowii* and *Fusarium lichenicola* on Total Petroleum Hydrocarbon in an Oilfield Wastewater in Rivers State

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Authors' contributions

This work was carried out in collaboration between both authors. Author WJO designed the study, wrote the protocol and the first draft of the manuscript. Author AO managed the analyses of the study, performed the statistical analysis and literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

This study investigated the potential of *Aspergillus sydowii* and *Fusarium lichenicola* as mixed cultures in the biodegradation of Total Petroleum Hydrocarbons TPHs in oilfield wastewater. Oilfield wastewater was collected from an onshore oil producing platform and biodegradation of total petroleum hydrocarbons was investigated using standard methods. Fungi were isolated from oilfield wastewater contaminated soils obtained from the vicinity of the oil producing platform. Experimental control set-up and treatment with mixed culture of fungal isolates were periodically analyzed on days 7 and 21 intervals for total petroleum hydrocarbon degradation using Gas Chromatography (GC). The total amount of TPHs on day 1 recorded 381. 871 mg/l. The amount of TPHs on days 7 and 21 in the mixed culture of fungi was 108.975 mg/l and 21.105 mg/l respectively while TPHs in control was 342.891 mg/l and 240.749 mg/l respectively. There was a significant difference between the mixed culture and the control on days 7 and 21 at $p \leq 0.05$. The results therefore revealed actual and

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significant reduction of TPHs in the mixed culture. In addition, there was clearance of n-alkanes by the mixed culture. This suggests that fungi have great potentials in biodegradation of TPHs and in remediation of TPH contaminated environments.

Keywords: Oilfield wastewater; TPHs; biodegradation.

1. INTRODUCTION

Crude oil is a complex mixture of hydrophobic components such as n-alkanes, aromatics, resins, and asphaltenes. Some of these components may be toxic to microorganisms while some stimulate microbial activity especially at low concentrations [1]. Oil industrial activities include among others drilling, exploration and production processes. It also comes from oil-well blowouts, seepages and deballasting operations which results in the contamination of environment [2,3]. Obire and Wemedo [4] also reported sales and uses of crude oil and refined petroleum products as sources of petroleum hydrocarbons in the environment. Breaks in oil pipelines and pipeline overflow and stationary spills from storage tanks in refinery areas have also been documented. Rowell [5] also reported marine transportation, slicks on water, road transport and rail transport as various ways in which crude oil and refined products can gain access into the environment. Ekweozor and Ikomah [6] and Obire and Amusan [7] also reported deliberate discharge of oilfield wastewater or effluent as a source of environmental contaminant. Many technologies recently have come up with ways to manage hydrocarbon contamination. Bioremediation comprises of a set of technologies that aid in the removal of contaminants or render them less toxic using biological activities [8]. This can be achieved with the help of unique microorganisms such as bacteria, microalgae and fungi, either isolated from a different environment or introduced into the contaminated sites or by enriching the organisms already present at the contaminated sites [9]. Microbes utilize crude oil as carbon substrate and biodegrade the hydrocarbons into simpler substances such as carbon dioxide and water [10]. It is an environmentally friendly approach. The ubiquitous distribution of yeast and fungi and their ready isolation from oil-contaminated environments indicate that they play an important role in the degradation of oil spilled in the environment. This study is aimed at investigating the potentials of *Aspergillus sydowii* and *Fusarium lichenicola* isolated from oilfield wastewater enriched soils obtained from an oil producing platform in the biodegradation of total petroleum hydrocarbon.

2. MATERIALS AND METHODS

2.1 Collection of Oilfield Wastewater and Soil Samples

Oilfield wastewater was collected from Ogbogu Flow Station, an onshore oil production platform located in Ogba/Egbema/Ndoni local government Area (ONELGA) of Rivers State, Nigeria. The Oilfield wastewater samples were collected using four (4) liters capacity plastic bottles and stored in an ice packed cooler.

The soil samples were collected 80 meters away from the discharge pond at a depth of 0-15 cm with a sterile spatula into sterile polythene bags and stored in an ice packed cooler. The collected and appropriately labeled oilfield wastewater and soil samples were immediately transported to the laboratory for analysis within 24 hours for processing and analyses.

2.2 Soil Contamination with Oilfield Wastewater

Soil contamination with oilfield wastewater was carried out by inoculating various concentrations (0% - control, 10%, 25%, 50% and 75%) of oilfield wastewater into 100g of soil samples each and incubated in a rotary shaker. Samples were withdrawn at different time intervals or incubation periods.

2.3 Determination of *Aspergillus sydowii* and *Fusarium lichenicola* from Enriched Soils

An aliquot (0.1 mL) of serial dilution (10^{-2}) of each sample was plated onto separate Potato dextrose agar plates to which 0.1 mL of streptomycin solution was incorporated to suppress bacterial growth. The plates were incubated at 28ºC for 5-7 days and the discrete colonies that developed were molecularly identified as *Aspergillus sydowii* and *Fusarium lichenicola* using the internal transcribed spacer.

2.4 DNA Extraction of Fungi

Extraction was done using a ZR fungal/bacterial DNA mini prep extraction kit supplied by Inqaba South Africa. A heavy growth of the pure culture of the suspected isolates was suspended in 200 µL of isotonic buffer into a ZR Bashing Bead Lysis tubes, 750 µL of lysis solution was added to the tube. The tubes were secured in a bead beater fitted with a 2 mL tube holder assembly and processed at maximum speed for 5 minutes. (Ochogba, et al. 2017). The ZR bashing bead lysis tubes were centrifuged at 10,000X *g* for 1 min.

Four hundred µL of supernatant was transferred to a Zymo-Spin IV spin Filter (orange top) in a collection tube and centrifuged at 7000X *g* for 1 min. One thousand two hundred µL of fungal/bacterial DNA binding buffer was added to the filtrate in the collection tubes bringing the final volume to 1600 µL, 800 µL was then transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000X*g* for 1 min, the flow through was discarded from the collection tube. The remaining volume was transferred to the same Zymo-spin and spun. Two hundred µL of the DNA Pre-Was buffer was added to the Zymo-spin IIC in a new collection tube and spun at 10,000X*g* for 1 min followed by the addition of 500 µL of fungal/bacterial DNA Wash Buffer and centrifuged at 10,000X*g* for 1 min.

The Zymo-spin IIC column was transferred to a clean 1.5 µL centrifuge tube, 100 µL of DNA elution buffer was added to the column matrix and centrifuged at 10,000X*g* µL for 30 sec to elute the DNA. The ultra pure DNA was then stored at -20ºC for other downstream reaction.

2.5 DNA Quantification of Fungi

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was launched by double clicking on the Nanodrop icon. The equipment was initialized with 2 µL of sterile distilled water and blanked using normal saline. Two µL of the extracted DNA was loaded onto the lower pedestal the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the “measure” button.

2.6 Internal Transcribed Spacer (ITS) Amplification of Fungi

The ITS region of the rRNA genes of the isolates was amplified using the ITS1F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4: 5'- TCCTCCGCTTATTGATATGC-3, primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 µL for 35 cycles. The PCR mix included: the X2 Dream *Taq* Master mix supplied by Inqaba, South Africa (*Taq* polymerase, dNTPs, $MgCl_2$), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 min; denaturation, 95ºC for 30 sec; annealing, 53ºC for 30 sec; extension, 72ºC for 30 sec for 35 cycles and final extension, 72ºC for 5 min. The product was resolved on a 1% agarose gel at 120V for 15 min and visualized on a blue light transilluminator.

2.7 Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10 µL, the components included 0.25 µL BigDye® terminator v1.1/v3.1, 2.25 µL of 5 x BigDye sequencing buffer, 10uM Primer PCR primer, and 2-10 ng PCR template per 100 bp. The sequencing condition were as follows 32 cycles of 96ºC for 10 sec, 55ºC for 5 sec and 60ºC for 4 min.

2.8 Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using Clustal X. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 [11]. The bootstrap consensus tree inferred from 500 replicates [12] is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method [13].

2.9 Biodegradation Studies

Aspergillus sydowii and *Fusarium lichenicola* isolates were sub cultured on sterile potato dextrose agar medium and incubated for 3-5 days at ambient temperature. A loopful of each

fungi was incubated into 4 mL of potato dextrose broth and incubated for 3-5 days at ambient temperature, thereafter, transferred to 250 mL conical flasks containing 50mL of sterile defined mineral salts medium (MSM) and incubated for 3-5 days at ambient temperature.

The experimental set up labeled A1 and A2 in a flask contained 125 mL of oilfield wastewater (OFWW) each. Flask A2 was inoculated with 6.25 ml mineral salt medium containing the *Aspergillus sydowii* and *Fusarium lichenicola*. The control, A1 had the oilfield wastewater only. The total petroleum hydrocarbons were analyzed on days 1, 7 and 21 using Gas Chromatography. Flame Ionization Detector GC/FID.

2.10 Determination of Total Petroleum Hydrocarbon

Residual total petroleum hydrocarbon (TPH) was extracted from the samples and quantified using Gas Chromatography- Flame Ionization Detector (GC-FID). The analysis was carried out using a Schimadzu GC-17A Gas Chromatography equipped with flame ionization detector. Samples were extracted using liquid-solid and liquid-liquid extraction methods respectively. A DB-I column was used with the following dimensions 30 m × 0.2 mm; 0.25 µm film thickness; 0.32 i.d. Helium was the carrier gas at a flow rate of 1 ml/ min. Analyses were carried out in split injection mode using a split ratio 5:1. The injection port was set at 250ºC. The oven temperature was programmed from 40ºC for 10 min, the 20ºC per min to 330ºC, holding this temperature for 10 min. Separation occurs as the vapour constituent partition between the gas and the liquid phases. The samples were automatically detected as it emerges from the column by the FID detector.

3. RESULTS

The total petroleum hydrocarbon on day 1 was 381.871 mg/L. On day 7, the control recorded 342.891 mg/l total petroleum hydrocarbon remaining while day 21 recorded 240.750 mg/ L as shown in Table 1. The set up with *Aspergillus sydowii* + *Fusarium lichenicola*, as seen in Table 1 recorded 108.975 mg/ L and 21.105 mg/ L on days 7 and 21 respectively. The percentage removal of the control on day 21 was 36.9% while the set up containing *Aspergillus sydowii* + *Fusarium lichenicola* recorded 94.5%.

The following n-alkanes present on day 1 were: C_8, C_9, C_{10}, C_{12}, C_{14}, C_{15}, Pr, C_{18}, C_{19}, C_{20}, C_{22}, C_{23} and C_{26} as shown in Fig. 1. There was no clearance of the n-alkanes by the control as shown in Figs. 2 and 3. Day 7 treatment option (*Aspergillus sydowii* + *Fusarium lichenicola*) cleared C_{12} and C_{22} while all the n- alkanes were cleared on day 21 except C_9 and C_{14} (Fig. 5).

Table 1. Biodegradation of Total Petroleum Hydrocarbon (TPH)

Treatments	Day 1 (mg/ L)	Day 7 (mg/ L)	Day 21 (mg/ L)	% Removal
Control (OFWW)	381.871	342.891	240.750	36.9
Aspergillus sydowii + *Fusarium lichenicola* + OFWW	381.871	108.975	21.105	94.5

Key: OFWW = Oilfield wastewater

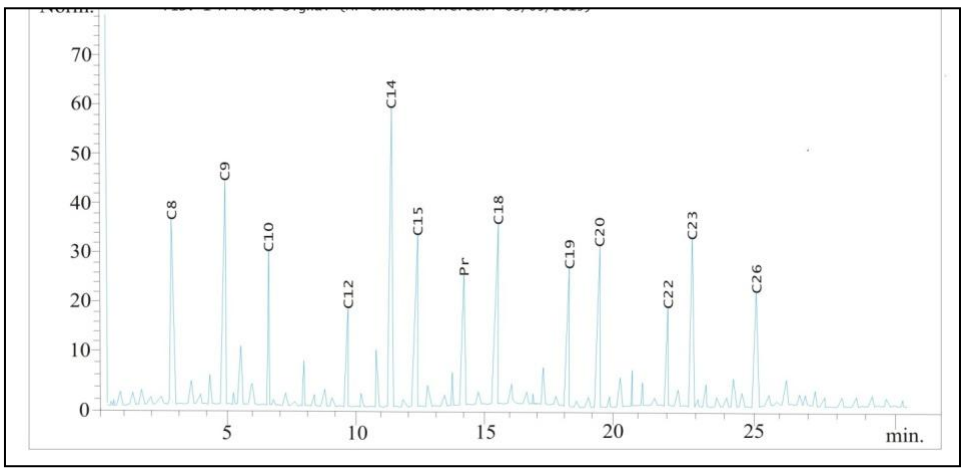

Fig. 1. GC profile showing the Total Petroleum Hydrocarbon (TPH) of the control on day 1

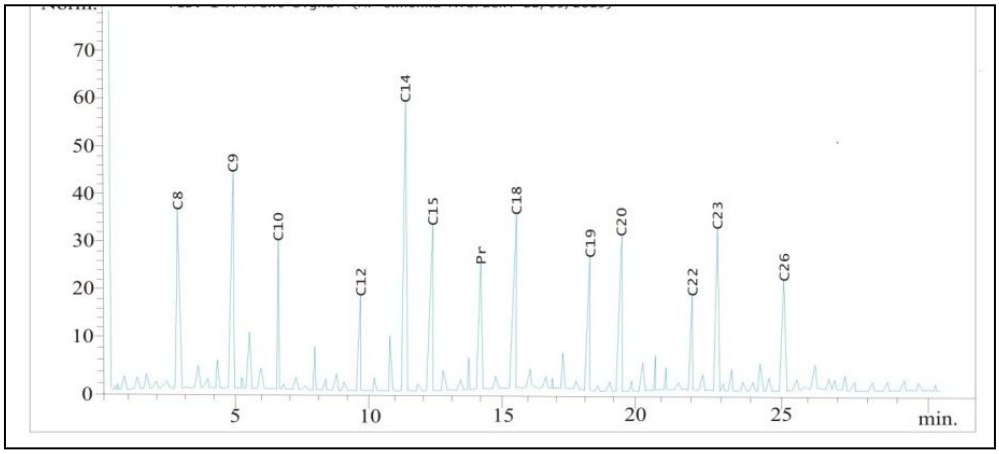

Fig. 2. GC profile showing the biodegradation of Total Petroleum Hydrocarbon (TPH) by the control on day 7

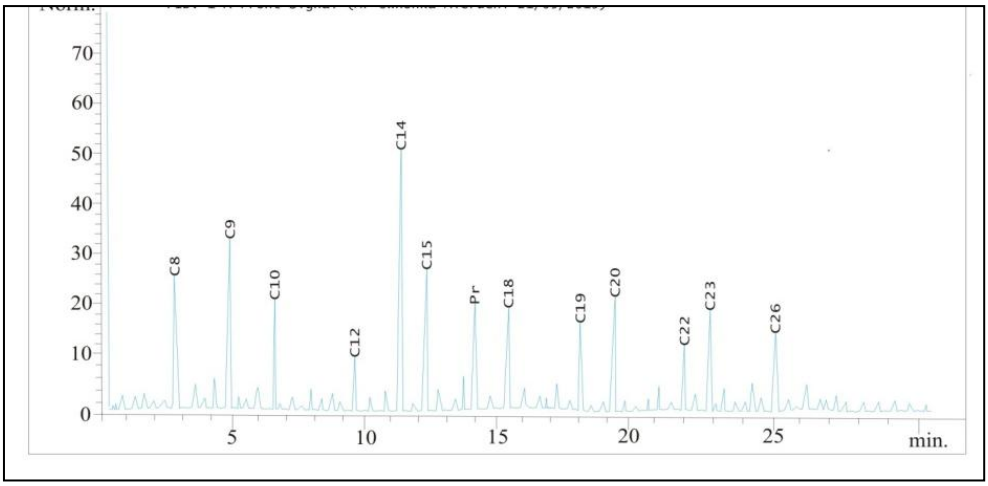

Fig. 3. GC profile showing the biodegradation of Total Petroleum Hydrocarbon (TPH) by the control on day 21

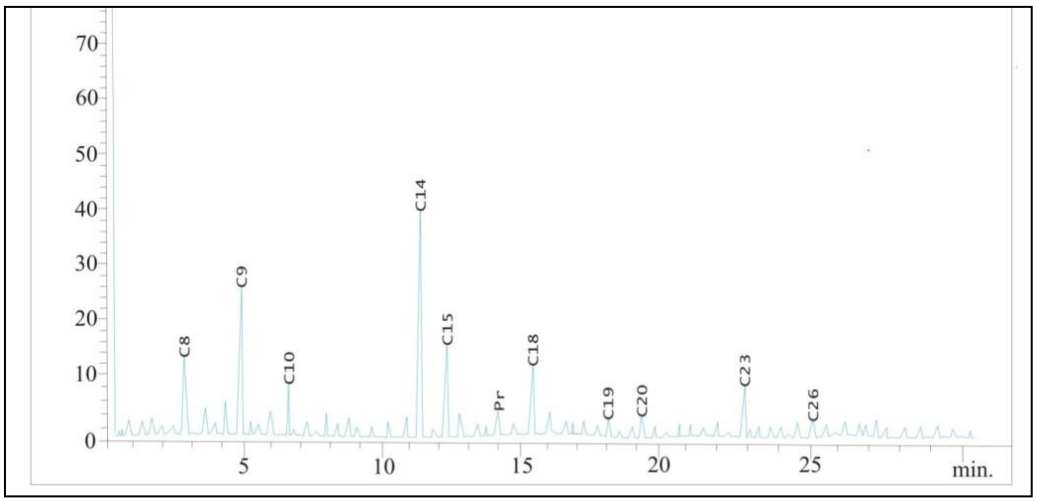

Fig. 4. GC profile showing the biodegradation of TPH by *Aspergillus sydowii* + *Fusarium lichenicola* on day 7

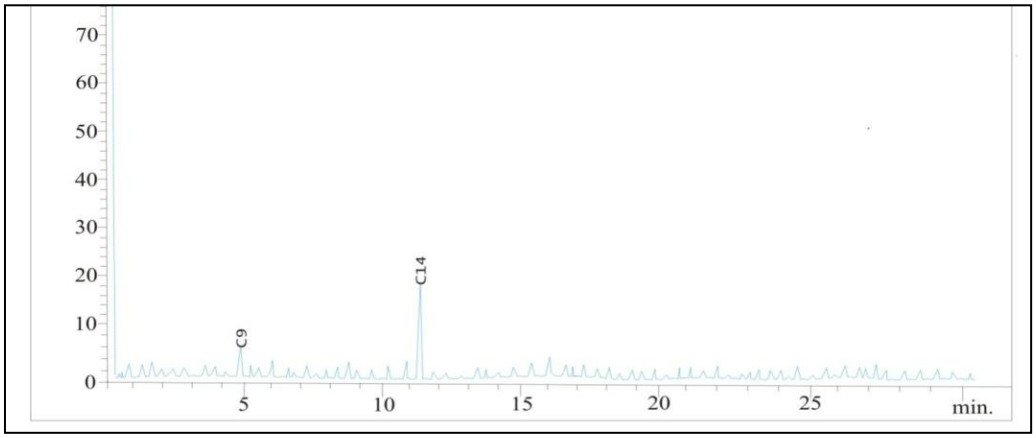

Fig. 5. GC profile showing the biodegradation of TPH by *Aspergillus sydowii* + *Fusarium lichenicola* on day 21

4. DISCUSSION

Biodegradation process is the major natural mechanism for elimination of oil from the environment but it occurs at a distressingly slow rate. Speeding up this process is the ultimate aim of recent research projects. Fungi plays important role in the degradation of oil spilled in the environment because they are ubiquitous and are readily isolated from oil-contaminatedsites. Obire [14] reported *Candida, Rhodotorula, Saccharomyces* and *Sporobolomyces* species and the moulds, *Aspergillus niger, Aspergillus terreus, Blastomyces* sp., *Botryodiplodiatheo bromae, Fusarium sp., Nigrospora sp., Penicillium chrysogenum, Penicillium glabrum, Pleurofragmium* sp., and *Trichoderma harzianum* as oil degraders in aquatic environment. Hydrocarbon degrading fungal genera isolated and reported by Bartha and Atlas [15] were *Aspergillus, Aureobasidium, Candida, Cephalosporium, Cladosporium, Cunningamella, Hansenula, Penicillium, Phodosporidium, Rhodotorula, Saccharomyces, Sporobolomyces, Torulopsis* and *Trichosporon.*

The fungi used in the biodegradation study were molecularly identified as *Aspergillus sydowii* and *Fusarium lichenicola* and were submitted to GenBank with accession numbers MN094361 and MN094360 respectively. The amount of total petroleum hydrocarbon removed on days 7 and 21 in the treatment options were significantly different at $p<0.05$ from the removal in the control. The total petroleum hydrocarbon utilization potential by the mixed culture of *Aspergillus sydowii* and *Fusarium lichenicola* showed higher percentage removal (94.5) than the control (36.9). Studies have shown that various hazardous contaminants can be degraded by fungi, including *Aspergillus* sp. and *Fusarium* sp. [16]. The n-alkanes in the treated option revealed clearance of many peaks on day 21 compared to untreated, which indicates that they were utilized by the mixed culture fungi. This result was similar to the findings of George, et al. [17]. The greater capacity to remove individual total petroleum hydrocarbon during the incubation period could be attributed to the adaptation of these fungi to the pollutant composition as well as the enzymatic systems associated with the fungi.

5. CONCLUSION

The result revealed high removal of total petroleum hydrocarbon by the mixed culture of *Aspergillus sydowii* and *Fusarium lichenicola* isolated from enriched soil from the oil production vicinity. Therefore, they can be considered as key components in the clean up strategy for oilfield wastewater contaminated with total petroleum hydrocarbon.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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